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# Characterization of a Native Polysaccharide Hapten from *Brucella melitensis*

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**The $^{13}C$ nuclear magnetic resonance spectrum of *Brucella melitensis* native polysaccharide hapten proved to be very similar to that generated by the O-specific chain (PS) isolated from *B. melitensis* lipopolysaccharide; that is, to a linear polymer in which the repeating unit is composed of five *N*-formylperosaminyl residues, one of them being substituted at position C-3 and the other four at position C-2. The serological analysis suggests that the so-called A determinant is present solely in *Brucella abortus* PS, the M determinant is only in *B. melitensis* PS, and the extensive cross-reaction observed is due to a determinant shared by both polysaccharides.**

*Brucella melitensis* polysaccharide haptens, isolated from either the rough B115 (PB) or the smooth 16M (NH) strain, are valuable in the differential diagnosis of brucellosis (6, 8, 10, 13, 22, 23). A simple radial immunodiffusion assay with PB or NH incorporated in the gel distinguishes infected from vaccinated cattle; sera from infected animals produce a ring of precipitation around the well after 3 h of incubation. An advantage over other serological tests is that this technique can be applied in large-scale testing in adult, vaccinated herds as early as 2 months after vaccination, when postvaccinal agglutinins and complement-fixing antibodies may still be present at diagnostically significant levels (6, 13). In previous work (10, 22, 23), it was demonstrated that these haptens cross-react with the O-specific chain (PS) of the lipopolysaccharide (LPS) and with the *Yersinia enterocolitica* O:9 native hapten. The structures of the PS-chains of *Brucella abortus*, *B. melitensis*, and *Y. enterocolitica* LPS have been elucidated (1, 3–5, 16; A. M. Wu, C. G. Adams, and N. E. MacKenzie, Abstr. Int. Symp. Mol. Immunol. Complex Carbohydr., 1984, p. 20); the PS of *B. abortus* and that of *Y. enterocolitica* is a linear homopolymer of α-1,2-linked 4,6-dideoxy-4-formamido-D-mannopyranosyl units (*N*-formylperosamine), while the PS of *B. melitensis* is a linear polymer made up of repeating units composed of five linked *N*-formylperosaminyl residues, four being α-1,2 linked and one α-1,3 linked. In this respect, the elucidation of the structures of PB and NH is of great interest, since it contributes to the understanding of the differential reactivity of antibodies from infected and vaccinated animals, as well as of the relationships and cross-reactivities of these molecules.

NH from *B. melitensis* 16M, PB from rough *B. melitensis* B115, and NH from *Y. enterocolitica* O:9 were isolated from the soluble cytoplasmic fractions as described elsewhere (6, 8, 10, 13, 22). The crude polysaccharides were contaminated with nucleic acids, glucans, and various amounts of protein (22, 23). Further purification was carried out by treatment of 50 mg of these materials per ml with 20 μg of DNase and 20 μg of RNase A (Sigma Chemical Co.) per ml in 0.15 M phosphate-buffered saline (PBS), pH 7.0, for 15 h at 25°C, followed by digestion with 20 μg of proteinase K (Merck & Co., Inc.) per ml in PBS for 24 h at 25°C and three cycles of centrifugation at 106,000 × *g* for 3 h. The supernatants were pooled, dialyzed, and lyophilized. The remaining glucans contaminating the NH preparation (22) were completely removed by filtration through a column of Bio-Gel P300 (Bio-Rad Laboratories) (23). The purified NH was free of fatty acids, 2-keto-deoxyoctonate, nucleic acids, and protein, as evidenced by colorimetric assays, gas-liquid chromatography, and analysis on the amino acid analyzer by procedures described previously (20, 22). Traces of glucose and mannose in the NH preparation were detected by gas-liquid chromatography. PSs were obtained by acetic acid hydrolysis from *B. melitensis* 16M and *B. abortus* 1119 LPSs and purified as described previously (22). None of the polysaccharide preparations entered in sodium dodecyl sulfate-polyacrylamide gel electrophoresis coated erythrocytes, had mitogenic or immunogenic activities, or reacted with rabbit sera against *Brucella* proteins or with anti-rough LPS or anti-lipid A antibodies, by procedures described elsewhere (13, 18–23).

$^{13}C$ nuclear magnetic resonance (NMR) analysis, carried out on a Bruker FT spectrometer WM300 at 75.47 MHz as described by Caroff et al. (4, 5), and immunoelectrophoresis (21) confirmed that the NH preparations were free of detectable contaminants. Rabbit immunoglobulin G against protein-free *B. abortus* and *B. melitensis* smooth LPS (14) was produced, isolated, and then conjugated to horseradish peroxidase, as described previously (20, 22, 23). Immunodiffusion tests and enzyme-linked immunosorbent assays (ELISA) were performed as reported elsewhere (20–23).

The $^{13}C$ NMR spectrum generated by *B. melitensis* NH (Fig. 1) is practically identical to that obtained with the *B. melitensis* PS (1, 16, 19), but it differs from those produced by the PSs of *B. abortus* and *Y. enterocolitica* O:9 (1, 3–5; Wu et al., Abstr. Int. Symp. Mol. Immunol. Complex Carbohydr., 1984) by a second set of peaks with $^{13}C$ NMR signals at 102.72, 77.20, 69.86, 51.99, and 18.31 ppm, indicating the presence of an additional sugar constituent. This sugar must be identical or closely related to *N*-formylperosamine and is probably substituted at position C-3, as indicated by the 102.72-ppm signal for the anomeric carbon

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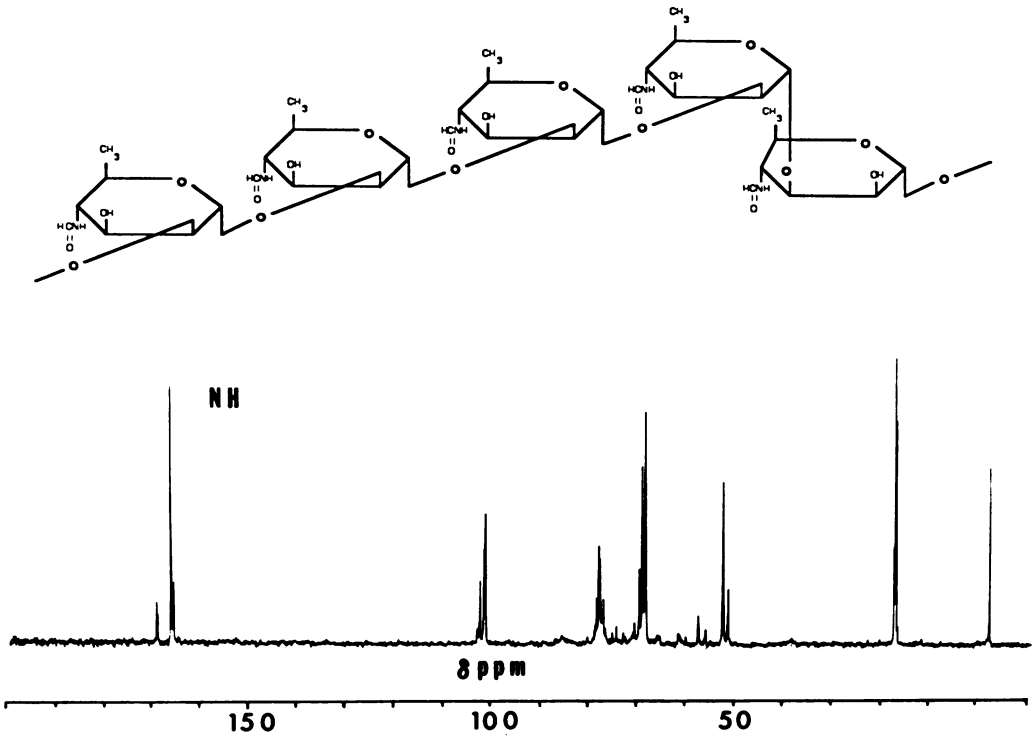


FIG. 1. $^{13}C$ NMR spectrum of *B. melitensis* 16M NH. The model of the pentasaccharide unit is based on the work of L'vov et al. (16) and Bundle et al. (1).

(24). The characteristic coupling constant ($^1J_{C-H}$ = 173 Hz) observed for the anomeric carbon shows that this sugar is also α linked. On the basis of reported data for the PS structure (1, 16) and on the basis of our results with NH, we conclude that the basic structure of *B. melitensis* NH is identical to that of the PS from *B. melitensis* LPS, that is, a linear polymer which is composed of a repeating block of five *N*-formylperosaminyl residues. One of the sugars is substituted at position C-3, and the other four are substituted at C-2 (Fig. 1).

Results of the double-immunodiffusion assay of different polysaccharides reacted with nonabsorbed rabbit serum against smooth *B. abortus* LPS are presented in Fig. 2. The optimal precipitation reaction occurred at 4 h of incubation; after this time, the bands diffused closer to the antibody well until their complete disappearance. Under these conditions, spur formation is difficult to observe. A competitive ELISA was therefore carried out, which permits determination of the extent of specific cross-reactions (Table 1). Unabsorbed anti-*B. abortus* and anti-*B. melitensis* sera strongly reacted with both purified LPS molecules attached to the plastic wells. On the other hand, exhaustive absorption of antisera with the heterologous killed bacteria generated highly monospecific sera which reacted weakly in immunodiffusion tests with antigen of the homologous serotype but did not precipitate with antigen of the heterologous serotype (*Y. enterocolitica* O:9 NH was used as the *B. abortus* serotype). The optical densities in the ELISA of the absorbed antisera decreased to background levels when reacted with the heterologous LPS. Absorption of anti-*Brucella* antisera considerably reduced the agglutinating titer against the complementary brucellae; the anti-*B. abortus* titer was lowered from 1/1,200 to 1/250, and the anti-*B. melitensis* titer decreased from 1/1,800 to 1/400. In addition, the homologous polysaccharides effectively competed for the antibody site in the ELISA when added together with the cognate antiserum (Table 1).

The usefulness of *B. melitensis* PB and NH in the serological differentiation of infected from vaccinated cattle has been extensively documented (6, 8, 10, 13, 22, 23). Unfortunately, the production of PB is not a stable characteristic of rough *B. melitensis* B115 (8), being very difficult to obtain

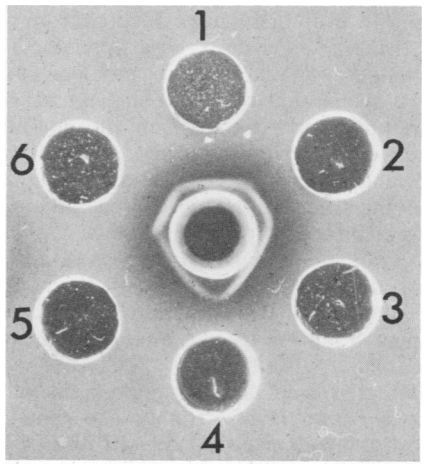


FIG. 2. Immunodiffusion analysis of *Brucella* and *Yersinia* polysaccharides in 0.8% agarose. NH (250 μg/50 μl) from *Y. enterocolitica* O:9 (well 1), NH (250 μg/50 μl) from *B. melitensis* 16M (well 2), PB (250 μg/50 μl) from *B. melitensis* B115 (well 3), PBS (well 4), PS (250 μg/50 μl) from *B. melitensis* 16M (well 5), and PS (250 μg/50 μl) from *B. abortus* 1119 (well 6) were reacted with serum from a rabbit immunized with *B. abortus* 1119 protein-free LPS.

TABLE 1. Competitive ELISA of anti-*Brucella* sera with different polysaccharides[a]

| Antiserum to: | *B. abortus* LPS | | | | | | *B. melitensis* LPS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Competition with: | | | | | | Competition with: | | | | | |
| | O chains | | | Polysaccharide haptens | | | O chains | | | Polysaccharide haptens | | |
| | Con | PSA | PSM | NHM | PB | NHY | Con | PSA | PSM | NHM | PB | NHY |
| *B. abortus* | 670 | 50 | 240 | 210 | 270 | 41 | 453 | 55 | 51 | 38 | 43 | 21 |
| *B. melitensis* | 581 | 37 | 15 | 27 | 30 | 48 | 730 | 333 | 12 | 12 | 26 | 310 |
| *B. abortus*, absorbed[b] | 170 | 10 | 207 | 185 | 167 | 19 | 26 | 41 | 18 | 16 | 19 | 7 |
| *B. melitensis*, absorbed[b] | 31 | 40 | 17 | 27 | 30 | 18 | 210 | 232 | 6 | 13 | 24 | 203 |

[a] The competitive assay was carried out by incubating 10 mg of the respective polysaccharide per ml with the serum diluted 1:250 in PBS for 24 h at 4°C before the ELISA. LPS (10 μg/ml) was adsorbed in the wells. Con, Control; PSA, O-specific chain from *B. abortus* 1119; PSM, O-specific chain from *B. melitensis* 16 M; NHM, native polysaccharide hapten from *B. melitensis* 16M; PB, polysaccharide hapten from *B. melitensis* B115; NHY, native polysaccharide hapten from *Y. enterocolitica* 0:9. Values are the optical density at 490 nm multiplied by 1,000.

[b] Antiserum absorbed with heterologous *Brucella* spp. by the method of Jones (12).

in quantities sufficient for chemical analysis. However, from a serological and biological point of view, both polysaccharides are indistinguishable (8, 10, 22), and it is probable that PB and NH are also chemically identical. On the other hand, in the immunodiffusion test *B. abortus* NH shows a precipitin reaction of partial identity with *B. melitensis* polysaccharides and in ELISAs exhibits different serological behaviors with sera from infected and vaccinated bovines (22) than LPS or PB. Since in the immunodiffusion test, the NHs of *B. abortus* and *Y. enterocolitica* O:9 give a precipitin reaction of total identity with the PSs of *B. abortus* and *Y. enterocolitica* (10), it can be assumed that the NHs of both bacteria have the same basic chemical structure, which is again similar to the PS (a linear homopolymer of *N*-formylperosamine residues).

It is difficult to explain why, despite their basic structural similarities, the NH and the PS moieties of the LPS have strikingly different serological behaviors. While the former can differentiate between infected and vaccinated cattle in the radial immunodiffusion test, the latter cannot. Interestingly enough, it has been demonstrated that when the NH is synthetically acylated (for adherence to plastic), it has a sensitivity and specificity in an ELISA similar to those of the purified LPS (23). Both distinguish infected from vaccinated cattle, with the efficiency depending upon the time lapse between vaccination and bleeding for serological testing. In this respect, it seems that close similarities in the chemical structures are apparently not sufficient but that other physical properties, such as molecular size, solubility, adequate presentation, and state of aggregation (23), are also important factors in the differential antibody recognition. We must also take into consideration that in contrast to the NHs, the *Brucella* PSs contain significant quantities of 2-keto-deoxyoctonate and quinovosamine (4, 22). Furthermore, depending upon the time, temperature, and pH of the acetic acid hydrolisis, there would be varying amounts of degradation of the PSs.

We also detected a polysaccharide hapten, which is difficult to purify since it is strongly associated to LPS. Although this molecule cross-reacts with the PS, it can be distinguished from the LPS by its serological and biological behavior (22), and it differs from the NH described here in that it possesses small quantities of lipid, coats erythrocytes, and can be adsorbed to plastic plates (unpublished results). In this respect, it might be that as in the case of enterobacterial common antigen (11), this form of *Brucella* polysaccharide hapten is bound to a lipid different from the lipid A (e.g., L-glycerophospatidyl residue) and is present on the surface of the bacteria as originally proposed by Diaz et al. (7).

For many years, it has been proposed that the A and M specificities of smooth *Brucella* spp. are found in a single complex present in the smooth LPS molecules but in different proportions (7, 17, 25). In our opinion, this concept is not totally valid since monospecific antisera (absorbed with the cognate LPS) do not react with the PS or NH of the heterologous serotype. Based on these results, as well as on the chemical analyses and the serological data with polyclonal and monoclonal antibodies (2, 3, 15, 22; J. T. Douglas and T. M. Buchanan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B29, p. 22), it is evident that the specificity responsible for the cross-reaction is a common determinant, as proposed in the earlier work by Douglas and Buchanan and by one of us (18), and that the A and M determinants are present only in *B. abortus* and *B. melitensis* LPS, respectively. We have to keep in mind, however, that this explanation is valid for the LPS molecule but not for the complete bacteria, since it has been established that some *Brucella* biotypes (e.g., *Brucella suis* 4 and *B. melitensis* 3) possess in their outer membranes both A and M determinants. One of those might be present in a structure different from that of the LPS molecule (e.g., as a glycolipid), or as recently suggested by Dubray and Limet (9), there may be A and M LPSs present at different ratios on the surfaces of cells from the same *Brucella* biovar. In addition, we examined only the PS from the LPS extracted in the phenol phase, ignoring some of the cross-reacting polysaccharides and LPS also present in the aqueous phase (18, 21, 22).

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